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PHOTOBOTREATMENT: INFLUENCE OF NITROGEN AND PHOSPHORUS RATIO IN WASTEWATER ON GROWTH KINETICS OF SCENEDESMUS OBLIQUUS

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PHOTOBIO-TREATMENT: INFLUENCE OF NITROGEN AND PHOSPHORUS RATIO IN WASTEWATER ON GROWTH KINETICS OF *SCENEDESMUS OBLIQUUS*

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*Nitrogen and phosphorus concentration in the effluent of a wastewater treatment plant can vary significantly, which could affect the growth kinetic and chemical composition of microalgae when cultivated in this medium. The aim of this work was to study the rate of growth, nutrient removal and carbon dioxide biofixation as well as biomass composition of *Scenedesmus obliquus* (S. obliquus) when it is cultivated in wastewater at different nitrogen and phosphorus ratio, from 1:1 to 35:1. A more homogeneous method for calculating productivities in batch reactors was proposed. The proper N:P ratio for achieving optimum batch biomass productivity ranged between 9 and 13 (263 and 322 mg L⁻¹ d⁻¹ respectively). This was also the ratio range for achieving a total N and P removal. Above and below this range (9–13) the maximum biomass concentration changed, instead of the specific growth rate. The maximum carbon dioxide biofixation rate was achieved at N:P ratio between 13 and 22 (553 and 557 mg CO₂ L⁻¹ d⁻¹ respectively). Lipid and crude protein content, both depend on the aging culture, reaching the maximum lipid content (34%) at the lowest N:P (1:1) and the maximum crude protein content (34.2%) at the highest N:P (35:1).*

KEY WORDS: *scenedesmus obliquus*, n:p ratio, photobiotreatment

INTRODUCTION

In recent years, microalgae have attracted the attention of scientists all over the world due to several reasons. Firstly, microalgae are considered nowadays as a promising source of high valuable products as well as feedstock for biofuels (Brennan and Owende 2010; Stephens *et al.* 2010). Microalgae could be an alternative source of vegetable oils as they have higher rates of biomass and oil production than terrestrial plants (Chisti 2007). Secondly, microalgae have the potential to reduce green house gases (GHG), especially carbon dioxide (CO₂) emissions through the CO₂ conversion into chemical energy via

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photosynthesis (Demirbras 2009). And finally, a major requirement of wastewater treatment is the need to remove high concentrations of nitrogen and phosphorus, which otherwise can cause a risk of eutrophication of the receiving water bodies. Many microalgae are able to grow in wastewater though their ability to assimilate nitrogen and phosphorus from those complex media. Extensive works have been conducted to explore the feasibility of using microalgae for wastewater treatment (Gonzalez *et al.* 1997; Aslan and Kapdan 2006; Ruiz-Marin *et al.* 2010; Ruiz *et al.* 2011; Arbib *et al.* 2012).

Regarding algal nutrition, carbon nitrogen and phosphorus are the main nutrient contributing to the microalgae biomass production. Nitrogen content of the biomass can range from 1% to more than 10%, depending on the species and the availability of nutrients (Grobbeelaar 2004). Carbon represents almost 50% content of the algae biomass while phosphorous represents less than 1% of algal biomass, being one of the most important growth limiting factors in algal biotechnology (Grobbeelaar 2004). A commonly assumed general composition for microalgae is the Redfield ratio ($C_{106}H_{181}O_{45}N_{16}P$) (Redfield, 1958). So, a culture medium with an N:P ratio of 16:1 (molar) would be required for an optimal growth. This formula is a point of departure to quantify possible nutrient limitation. It is noteworthy that many commercial nutrient solutions for growing microalgae are far from the proper N:P ratio proposed by Redfield. i.e., BG11 and Modified Allen's nutrient solutions have N:P ratio of 100:1. In those cases the nutrient solutions is phosphorus limited. From the other side, there are others commercial nutrient solution that are nitrogen limited, i.e., Bolds Basal and Zarrouck solutions with an N:P of 8.84 and 13.27 respectively.

The majority of urban wastewaters contain high concentration of nutrients and mainly nitrogen and phosphorus. Total nitrogen in domestic wastewater typically ranges from 20 to 70 mg L⁻¹ for low to high strength wastewater (Tchobanoglous *et al.* 2003). Influent concentration varies throughout the day and can vary significantly during rainfall events. Total phosphorus (TP) in domestic wastewater typically ranges between 4 and 8 mg L⁻¹ but can be higher depending on industrial sources. Typical concentrations of ammonia nitrogen and phosphates in secondary-treated wastewater fall into the ranges of 20–40 mg L⁻¹ and 1–10 mg L⁻¹, (McGinn *et al.* 2011), these concentrations imply N/P ratio (molar) between 9 and 44, which was really far from the theoretically of 16:1 (Redfield 1958). Therefore, this could affect the growth and the biomass composition of the microalgae, because at NP below 16 the system will be probably phosphorus limited, and thereby at NP above 16 it will be nitrogen limited. It is widely studied and reported that nutrient deficiencies of the culture media affect both the growth rate and the biomass composition (Ref).

Therefore the aim of this work was to study the effect of the variation on N:P ratio of the effluent from a conventional wastewater treatment plant on the growth rate, nutrient removal capability, carbon dioxide biofixation, lipid and crude protein accumulation of *S. obliquus*.

MATERIAL AND METHODS

Microalgal Strain

The microalga strain used in this study was *Scenedesmus obliquus* (SAG 276-10), obtained from the Culture Collection of Algae (SAG), Göttingen University (Germany). Inoculum for the experiments was cultivated in synthetic culture medium Combo (Kilham *et al.* 1998) at 20 ± 1°C and 250 μmol cm⁻² s⁻¹ light intensity under 14:10 light dark cycle.

Culture Media and Experimental Design

A pretreated urban wastewater sample was taken from the effluent of the WWTP of Arcos de la Frontera (25,000 inhabitants) (Latitude: 36.749°, Longitude: -5.793°, Spain), where the wastewater was submitted to pretreatment, primary settling, activated sludge and secondary settling. In the laboratory, the sample was filtered through 0.45 μm pore diameter glass fiber filter and autoclaved for 20 min at 1 kg cm^{-2} to assure axenic cultures. Wastewater nutrient concentration was: $\text{NO}_3\text{-N}$: 8 mg L^{-1} ; $\text{NO}_2\text{-N}$: 0.77 mg L^{-1} ; $\text{NH}_4\text{-N}$: 14 mg L^{-1} and $\text{PO}_4\text{-P}$: 5.69 mg L^{-1} .

Seven different sets of experiments were performed (Table 1), by changing the initial nitrogen and phosphorus ratio of the original wastewater (R_9). N:P ratio was increased by the addition of NH_4Cl and KNO_3 to the wastewater as nitrogen source at the same N- $\text{NH}_4\text{-N}/\text{N-NO}_3$ proportion that in the wastewater ($R_{13}/R_{22}/R_{35}$). Organic nitrogen and organic phosphate in the culture media were below detection limits (0.5 mg N L^{-1} and 0.044 mg P L^{-1}) concentration of organic nitrogen and $\text{NO}_2\text{-N}$ were very low, so it has not been taken into account. N:P decrease was done by adding K_2HPO_4 to the wastewater as phosphorus source ($R_1/R_3/R_5$).

Experimental Conditions

The experiments were conducted in 2000 ml Pyrex bottles. The culture was mixed and aerated with 0.2 μm pre-filtered air using a membrane air pump. 5% CO_2 enriched air was bubbled into the bottles from the bottom at a flow rate of 1 vvm ($\text{L}_{\text{air}}\text{L}_{\text{reactor}}^{-1}\text{min}^{-1}$). A set of 6 fluorescent lamps (3 Sylvania Gro-Lux F57W and 3 Philips TLD 58W) providing 250 $\mu\text{mol cm}^{-2}\text{s}^{-1}$ was used as light source, measured with a Hansatech QRT1 Quantitherm light meter. The tests were conducted in a climatic chamber under controlled temperature ($20 \pm 1^\circ\text{C}$) and a photoperiod of 14:10 light:dark. At the beginning of the experiment all reactors were inoculated with the same volume of pre-cultured microalgae in order to obtain similar initial concentration of biomass around 75 mg L^{-1} .

Analytical Methods

Microalgae biomass concentration was measured daily by optical density at 680 nm (OD_{680}). When necessary, samples were diluted appropriately to ensure values in the range of 0.1–1.0. In order to convert OD_{680} values to biomass as dry weight, a calibration curve was developed ($\text{SS (mg L}^{-1}) = 0.959 \cdot (\text{OD}_{680}) - 0.075$; $r^2 = 0.991$). Biomass dry weight as

Table 1 Nutrient composition of the different culturing media tested.

Reactors	$\text{NO}_3\text{-N (mg L}^{-1})$	$\text{NO}_2\text{-N (mg L}^{-1})$	$\text{NH}_4\text{-N (mg L}^{-1})$	$\text{PO}_4\text{-P (mg L}^{-1})$	pH	N:P (molar)
R_1	8.00	0.77	14.00	40.00	7.62	1.00
R_3	8.00	0.77	14.00	20.00	7.57	3.00
R_5	8.00	0.77	14.00	10.00	7.23	5.00
R_9	8.00	0.77	14.00	5.69	7.12	9.00
R_{13}	13.00	0.77	20.00	5.58	7.16	13.00
R_{22}	22.00	0.77	36.00	5.79	7.10	22.00
R_{35}	40.00	0.77	52.00	5.79	6.95	35.00

suspended solids, was determined gravimetrically according to Standard Methods (APHA, 2008).

Samples from each flask were taken daily to determine phosphorus and nitrogen concentration after filtration. Phosphorus was measured as orthophosphate (P-PO_4^{3-}) and nitrogen as the sum of nitrate and ammonium ($\Sigma\text{N:NO}_3^- - \text{N} + \text{NH}_4^+ - \text{N}$). Analysis of nitrate was carried out according to Spectroquant Cod. 1.14773.0001 (Merck). Ammonium and phosphates were analyzed according to Standard Methods 4500-NH₃ D, 4500-PE (APHA, 2008).

Once the cultures reached the stationary growth stage, biomass was harvested by means of centrifugation at 6000 rpm for 15 min (Centrifuge Mixtasel-BL Selecta®). The pellet was rinsed with distilled water, re-centrifuged three times and dried in a vacuum freeze-dryer (LABCONCO®) during 72 h. Biomass elementary analysis (percentage of C, N, H and S) were performed by a Leco® CHNS 932 analyzer.

The crude protein estimation was obtained by the multiplication of the biomass nitrogen content by the factor 6.25 (Ho *et al.* 2010; Arbib *et al.* 2012). Biomass lipid concentration was determined in duplicate. Lipids were extracted according to a modified method reported by Takagi *et al.* (2006) and Wiltshire *et al.* (2000). To 90 mg of lyophilized pellets, 12 ml of 2:1 trichloromethane:methanol and 0.6 g of analytical grade quartz were added and the mixture was sonicated in a bath (60 kHz; 360 W) for 90 min. Extraction was done twice and both extracts were mixed, centrifuged and filtered to ensure quartz separation. Filtrate was evaporated under reduced pressure in a rotary evaporator. The remainder was dried at 100–105°C for 12 h and weighed as total lipids.

Determination of Growth Kinetic Parameters

The Verlhust logistic kinetic model (Verlhust 1838) was used to model the experimental biomass concentration evolution at the reactors. The model is a substrate independent equation and can accurately describe biomass growth in different cultures conditions which occurs in many batch bioreactors (Gong and Lun 1996). According to the model, the microbial growth could be expressed as a sinusoidal curve as described by equation 1. Integrating this equation we get equation 2, where μ_{max} is the maximum specific growth rate (d^{-1}), X_{max} , X_o and X are the concentration of biomass (g L^{-1}) at an operation time equal to infinite, zero and t respectively.

The productivity is an important parameter to consider in microalgae culturing techniques, as it shows the capability of a reactor to produce biomass under specific operational conditions. Firstly, the overall productivity (P_o) was calculated according to the equation 3, where X_{max} (mg L^{-1}) is the maximum final concentration achieved at the end of the experiment, that is, the superior asinthetic values of the sinusoidal growth curve; X_o is the initial biomass concentration (mg L^{-1}); t_∞ is the time required to reach X_{max} ; and t_o is equal to zero. This is the most applicable equation for many authors (De Morais and Costa 2007a,b; Ho *et al.* 2010; Tang *et al.* 2011; Arbib *et al.* in press). This methodology of calculating productivities takes into account only the experimental data with no kinetic modelling process.

In order to estimate productivity with a standardize methodology, lag phase should be minimized or even not be considered, because these is an extremely variable phase that depends not only on the experimental conditions, but also on the experimental methodology like initial inoculum concentration used and how the inoculum was obtained. Therefore when lag phase is included in productivity calculations, great and unnecessary data dispersion between authors is promoted. As many guidelines to test biodegradability establish

(OECD 2004) lag phase is considered as the time lapsed until 10% of biodegradation has been reached. In this work, that could be comparable to an increase of 10% of initial biomass concentration in the reactor ($X_{10} = 1.1 \times x_0$). Hence, lag phase (t_{10} ;h) is considered as the time required to reach X_{10} . In the same manner, the stationary phase should be also minimized or even excluded, in this case stationary phase (t_{90}) is considered as the time needed to reach the 90% of the final maximum concentration ($X_{90} = 0.9 \cdot X_{max}$). Therefore, from equation 2 it can obtain an expression for time as function of biomass concentrations (equation 4), and from equation 4 it can obtain the time needed to reach the stationary phase (t_{90}) and the lag phase (t_{10}) (Equations 5 and 6). Supposing that productivity in the exponential growth period in batch photobioreactors, it could be approximated to equation 7. By combination of equations 5, 6, and 7 it obtains an expression that relates productivity with kinetic parameters (μ_{max} , X_{max} and X_0) (equation 8).

Determination of Carbon Dioxide Biofixation Rate

According to the method described by De Morais and Costa (2007a,b), the carbon dioxide biofixation rate (P_{CO_2}) was calculated using the equation 9. Where %C is the carbon content in dried biomass obtained by elementary analysis; P_b is the batch productivity; and MW_{CO_2} and MW_C are the molecular weight of CO_2 and C respectively.

Data Analysis

Data kinetic modeling was performed using the software STATISTICA V.6.0 (Statsoft company). The Quasi-Newton method estimation was used with a convergence criterion of 10^{-4} . Confidence interval for $p \leq 0.05$ (C.I.) was calculated using the equation 10 where, is the mean, standard deviation and n is the sample size.

RESULTS AND DISCUSSION

Effect of N:P Ratio on Biomass Growth

The growth curves of *S. obliquus* under different N:P ratio are shown in Figure 1. *S. obliquus* was able to grow satisfactorily in all the cultures media tested, and a typical evolution of growth in batch culture was observed for all the reactors.

The logistic Verlhust kinetic model showed a good adjustment (lines in Figure 1) to the experimental data, being R^2 ($p \leq 0.05$) higher than 0.980 in all cases (Table 2).

In Table 2 it can be appreciated that the lag phase in all the reactors was similar, t_{10} varying between 2.31 and 3.50 h for R_1 and R_{35} respectively (Table 2). Despite the fact that there were no significant differences in t_{10} , important differences between both groups were obtained for t_{90} (Table 2), with average values of 127.10 ± 5.02 h for $R_1 - R_9$ and 166.43 ± 12.27 h for $R_{13} - R_{35}$ (Table 2). The stationary phase was reached firstly in those reactors where the N:P ratio was lower than 9 (R_1 , R_3 , R_5 and R_9).

Regarding the maximum final concentration achieved (X_{max}) (Table 2), two groups can be differentiated with significant differences between them ($p < 0.05$). Firstly, those reactors with N:P ratio higher than 9 (R_{13} , R_{22} , R_{35}) and secondly, those with lower N:P ratio (R_1 , R_3 , R_5). This second group includes the original wastewater (R_0). The average X_{max} in those experiments with N:P higher than 9 was about 2560 ± 110 mg L⁻¹, while in the case of N:P below 9, the average X_{max} was around 1585 ± 8.86 mg L⁻¹ (Table 2)

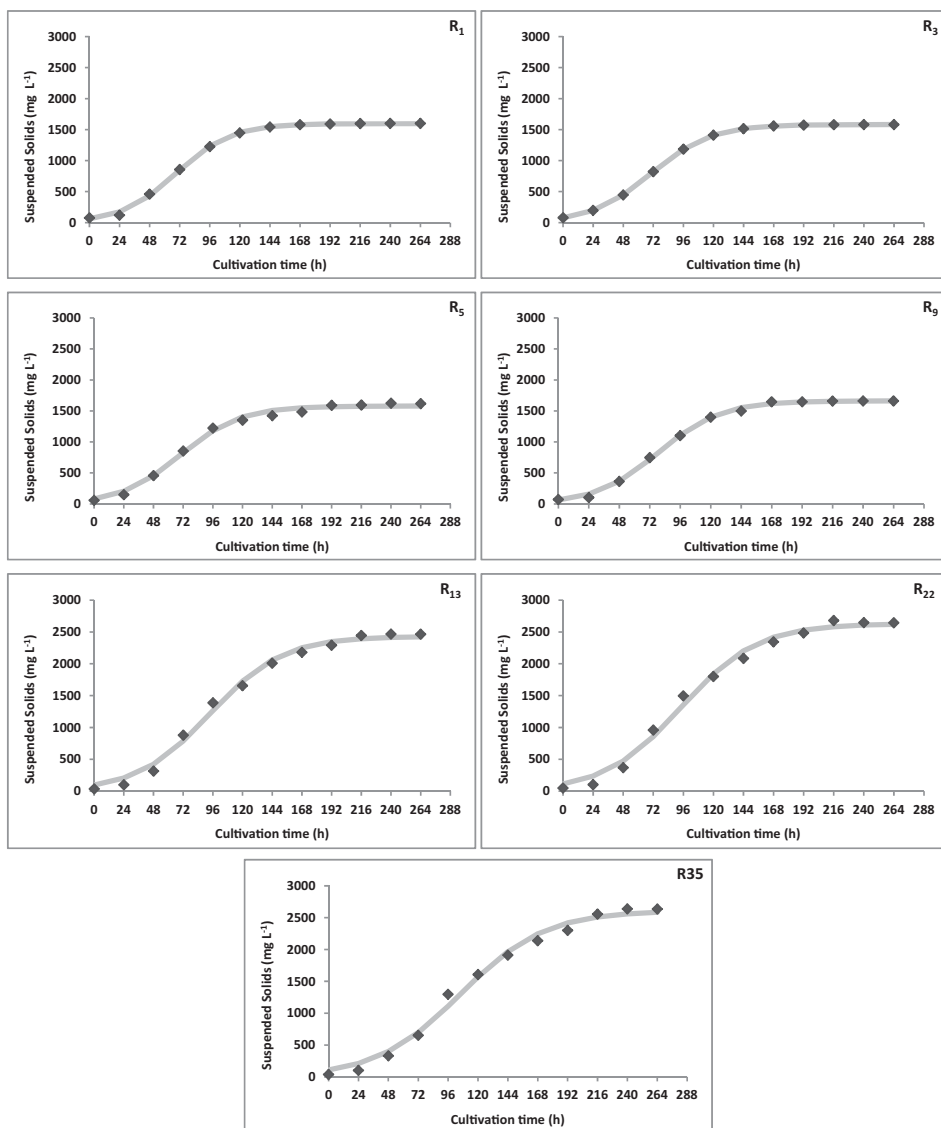


Figure 1 Growth curves of *S. obliquus* in different cultures media tested. Lines represent the predicted data and dots the experimental ones.

and similar that in the reactor with original wastewater (R_9 , $1661 \pm 32 \text{ mg L}^{-1}$). The increase of nitrogen concentration (higher N:P ratios) enhances X_{max} , while phosphorous increase does not suppose an enhancement of final biomass concentration. This means that nitrogen could be considered the limiting nutrient in wastewater when N:P ratio is below 13. Therefore an important effect of the N:P ratio variation were observed in relation to the biomass generation capability.

In terms of growth rate (μ), there were no significant differences ($p < 0.05$) between reactors (Table 2). Except in the case of R_{35} where a significant reduction (23.5%) can be

Table 2 Biomass kinetic parameter for *S. obliquus* obtained by Verhulst logistic model for the different N:P ratio tested. X_{max} (mg SS·L⁻¹): Maximum final biomass concentration; μ_{max} (d⁻¹): Maximum specific growth rate; t_{90} (h): time to reach 90% of X_{max} ; t_{10} (h): time to reach 110% of X_0 ; P_0 (mg·L⁻¹·d⁻¹): overall productivity; P_b (mg·L⁻¹·d⁻¹): batch productivity.

	X_{max} (mg L ⁻¹)	μ_{max} (d ⁻¹)	t_{10} (h)	t_{90} (h)	P_b (mg L ⁻¹ d ⁻¹)	P_o (mg L ⁻¹ d ⁻¹)	R ²
R ₁	1595.4 ± 21.0	1.104 ± 0.084	2.31	125	284	143	0.996
R ₃	1582.0 ± 51.0	1.014 ± 0.098	2.31	127	268	151	0.986
R ₅	1578.8 ± 51.0	0.984 ± 0.120	2.53	128	262	145	0.988
R ₉	1661.0 ± 32.0	0.984 ± 0.096	2.50	132	263	156	0.980
R ₁₃	2429.6 ± 114.2	0.820 ± 0.163	2.91	157	322	221	0.992
R ₂₂	2629.7 ± 135.7	0.790 ± 0.167	3.03	160	341	236	0.991
R ₃₅	2609.2 ± 160.0	0.709 ± 0.153	3.37	180	302	236	0.991

appreciated. In that case, such an extreme N:P ratio could provoke a slight stress on *Sc. Obliquus* population because of the excess of nitrogen. That stress due to extreme nutrient imbalance has also been appointed by (Grobeelaar 2004).

As it has been indicated in section 2.5, a standardize procedure for batch productivity calculations has been proposed. The differences between the traditional methodology (P_0) and the proposed one (P_b) can be observed in Table 2. In all the experiments P_b was higher than P_0 , because P_0 was calculated using the entire cultivation time (264 h), while in P_b the stationary and lag phase are not included. The maximum and minimum differences between both productivities proposed were reached in those reactors were the differences between total test time (264 h) and t_{90} were highest.

N:P ratio between 1 and 9 showed no significant differences in P_b (269.2 ± 10.13 mg L⁻¹ d⁻¹), and N:P ratio between 13 and 35 also showed no significant differences between them (321.70 ± 19.41 mg L⁻¹ d⁻¹). It is important to highlight that the differences in P_b appears at NP between 9 and 13, between this range a substantial increment in P_b occurs, being P_b at NP (13) 18.34% higher than NP (9), and at NP above 13 there were no significant increment in P_b . Therefore that indicates that the proper N:P ratio for achieving an optimum P_b for *S. obliquus* in wastewater ranged between 9 and 13. Those values were slightly lower than the proposed by the empirical formula for microalgae (C₁₀₆H₂₆₃O₁₁₀N₁₆P) (Redfield 1958),

When microalgae productivities values obtained in batch reactors by different authors are consulted, a great dispersion can be appreciated. For example, Rodolfi *et al.* (2009) and Tang *et al.* (2011) cultivated strains of *Scenedesmus* (*quadricauda* and *obliquus* respectively) in the same culture medium BG11, which was a phosphorus limited culture medium, N:P 100). Both obtained productivities lower than this study at high N:P ratio (35) (Table 2), 158 and 190 mg L⁻¹ d⁻¹ for Tang *et al.* (2011) and Rodolfi *et al.* (2009) respectively. On the other hand, Ho *et al.* (2012) cultivated *S. obliquus* in a nitrogen limited culture medium (modified Detmer's Medium, N:P 1.25) reporting a maximum biomass productivity of 440.68 ± 15.79 mg L⁻¹ d⁻¹, which was between 3 and 2.5 folds higher not only than those reported by Tang *et al.* (2011) and Rodolfi *et al.* (2009) but also higher than the productivities (both batch and overall) obtained in this study (Table 2). This dispersion of productivities between different authors are due to the wide variety of experimental conditions used: light intensity, light dark cycles, culture medium, species of microalgae, temperature, aeration, source and proportion of carbon dioxide, type of photobioreactor, etc. But one of these sources of variation lies in the way of calculating the productivity which is

strongly related with the variety of criteria used to stop the experiments. Some authors stops all microalgae culturing experiments at the same time or they do not indicate the criteria used to stop the test (De Morais and Costa 2007a,b; Ge *et al.* 2011; Tang *et al.* 2011) which conducts to productivities values (P_0) depending on the duration of the stationary phase (as occurs with R_I in this study). On the other hand some authors stop the experiments before the stationary phase is reached (Travieso *et al.* 2006; De Morais and Costa 2007a,b; Rao *et al.* 2007; Tang *et al.* 2011). In those cases the productivities are partial, as maximum biomass reached in the reactors is unknown. The use of a homogeneous, easy and realistic procedure for productivities calculation, as the proposed one, could contribute to the reduction of productivities data dispersion and facilitate comparison of data from different authors.

Effect of N:P Ratio on Nutrient Removal

The nitrogen and phosphorus removal capability of *S. obliquus* under different N:P ratios are shown in Figure 2. To compare nitrogen and phosphorus removal kinetics, the time required to reach 10 mg L^{-1} (ΣN) and 1 mg L^{-1} (P-PO_4^{3-}) (most restrictive concentrations in European Union Directive 98/15/CE concerning requirements of N and P in the effluents of the urban wastewater treatment) has been calculated. In order to obtain these times, phosphorous and nitrogen consumption has been modeled according to Quiroga-Sales kinetic model for substrate consumption by microorganisms in batch reactors (Equation 11) (Quiroga *et al.* 1999). Where, $S(\text{mg L}^{-1})$ is the substrate concentration in the medium (nitrogen or phosphorous) and K_2 , K_I , and K_0 are kinetic constants.

Nitrogen Removal

The experimental data (dots) corresponded well to the model applied for nutrient removal. In all reactors, R^2 was higher than 0.971 ($p < 0.05$) (Figure 2).

As can be observed in Table 3, at N:P ratio above 13 the minimum nitrogen removal efficiency were reached, 59 and 49% removal for R_{22} and R_{35} respectively. This could be due a phosphorous limitation, as in these tests phosphorous was totally removed (Table 3). On the other hand, under N:P values between 1 and 13 the average nitrogen removal was $91.6 \pm 2.19\%$. The maximum removal efficiency (95% in R_9) in this work was similar

Table 3 Nitrogen and phosphorus removal efficiency, time needed to reach 10 mg N L^{-1} (t_{10N}) and 1 mg P L^{-1} (t_{1P}) (most restrictive concentrations value in Directive 98/15/CE) for different N:P ratio.

	ΣN removal (%)	t_{10N} (h)	R^2	P removal (%)	t_{1P} (h)	R^2
R_1	89	110	0.989	16	—	0.911
R_3	91	106	0.986	25	—	0.974
R_5	92	104	0.996	52	—	0.995
R_9	95	90	0.995	100	110	0.965
R_{13}	91	138	0.995	100	115	0.986
R_{22}	59	*	0.975	100	131	0.984
R_{35}	42	*	0.971	100	145	0.991

Limit discharge not achieved

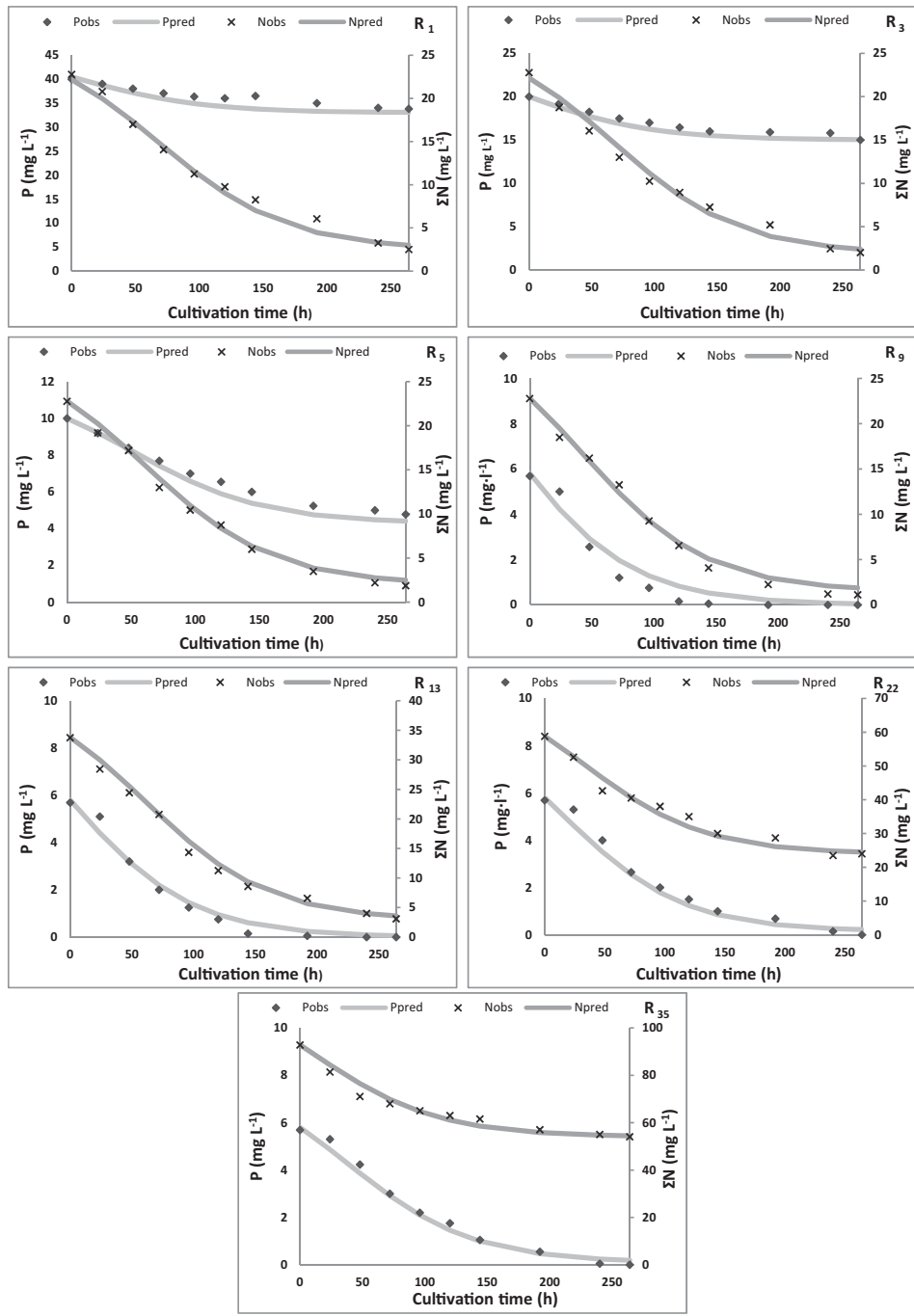


Figure 2 Time course of ΣN : (N-NO₃⁻ + N-NH₄⁺) and P- PO₄³⁻(mg L⁻¹) concentrations at different N:P ratio. Dots show the experimental data (obs: observed data) and lines the modelled data (pred: predicted data).

than that reported for *S. dimorphus* in diluted agro-industrial wastewater (N:P = 0.57) by Gonzalez *et al.* (1997).

Regarding to the nitrogen assimilation rate, at N:P ratio below 9 the nitrogen removal kinetic was similar (Table 3) being t_{10N} around 100 h. The lowest t_{10N} was reached in R_9 (90 h). At N:P of 13, the assimilation rate decreased 34.7% respect to R_9 . N:P above 13, t_{10N} is not even reached.

The proper N:P ratio for nitrogen removal is where we can get simultaneously high removal efficiency and low t_{10N} , in this case this value is around N:P = 9. Variations of N:P ratio on the wastewater affects greatly the nitrogen removal efficiency.

Phosphorus Removal

As for nitrogen, the experimental data of phosphorus consumption (Figure 2b) fitted well to the nutrient uptake kinetic model applied ($R^2 > 0.911$).

At N:P ratio below 9, phosphorus removal efficiency decreased drastically (Table 3) reaching the minimum in R_1 (16%). This fall in the removal efficiency could be due to a limiting nitrogen effect, as in those cases where phosphorous is totally removed (R_{22} and R_{35}) nitrogen is not totally removed while in those tests with N:P ratios bellow 9 (R_1 , R_3 and R_5), nitrogen is practically removed but not phosphorous.

On the other hand, at N:P ratio between 9 and 35 phosphorus assimilation rate increased as the ratio increased, reaching the minimum t_{1P} at R_9 (110 h) and the maximum at R_{35} (145 h). This difference can be related to the lowest specific growth rate achieved in R_{35} (Table 2) due to an excess in nitrogen, and therefore this excess may lead to a stress that affects the phosphorus uptake rate (Grobeelaar 2004).

Therefore, for an efficient simultaneous nutrient removal, a N:P ratio between 9 and 13 would be required. At this N:P ratio, both limits of discharge are reached (t_{1P} and t_{10N}) in a similar time (around 100 h). It is important to highlight that this is the same proper N:P ratio when the biomass production was the main goal. Xin *et al.* (2010) studied the effect of different nitrogen and phosphorus concentration on the growth rate of *Scenedesmus sp.* when it was cultivated in a synthetic modified BG11 growth medium. They concluded that for an efficiently nitrogen and phosphorus removal, an N:P ratio between 11 and 17 would be necessary. Those results were in agree with the results obtained in this study, where the culture medium employed was wastewater. In R_9 it could be appreciated that the phosphorus consumption was the slowest process (t_{1P} slightly higher than t_{10N}), and when the N:P ratio was increased from 9 to 13, the behavior changed. In this case t_{1P} was lower than t_{10N} , and both suffered an incensement respect to R_9 .

EFFECT OF N:P RATIO ON BIOMASS COMPOSITION

N:P Ratio Influence on the Carbon Dioxide Biofixation Capability

Analysis of carbon element showed that the carbon content of *S. obliquus* did not changed greatly when it is cultivated under different N:P ratio, been the average value for all the tests $44.92 \pm 0.48\%$ C (Table 4). Carbon dioxide biofixation rate P_{CO_2} (mg CO_2 L^{-1} d^{-1}) was calculated according to the equation 9 (De Moraes and Costa 2007a,b) and $P_{CO_2}^*$ according to the estimative method proposed by Chisti (2007) (Table 4). As shown in Table 4, there were no great differences between the carbon dioxide biofixation rate obtained by both methods, ranging between 6 and 13% for R_9 and R_{22} respectively,

Table 4 Carbon and nitrogen percentage (%) of dried weight (DW) biomass obtained by elementary analysis; Carbon dioxide biofixation rate (P_{CO_2}) and carbon dioxide requirement (Y_{CO_2}) of *S. obliquus* under different N:P ratio. Lipid and crude protein content (%) in the dry biomass.

	R ₁	R ₃	R ₅	R ₉	R ₁₃	R ₂₂	R ₃₅
C (%)	44.7±1.2	45.8±0.45	44.7±0.06	44.4±0.12	45.3±0.04	44.6±0.13	45.0±0.25
N (%)	1.8±0.03	1.9±0.02	1.8±0.01	1.9±0.01	4.8±0.01	5.2±0.05	5.5±0.06
P _{CO₂} (%)	421.3	442.7	420.7	471.9	535.3	557.4	498.3
P _{CO₂} * (mg CO ₂ L ⁻¹ d ⁻¹)	533.1	503.0	492.2	494.3	605.5	640.4	567
Y _{CO₂} (mg CO ₂ mg SS ⁻¹)	1.639	1.679	1.639	1.767	1.661	1.635	1.650
Lipid (%)	34	27	22	20	17	16	12
Protein (%)	11.4	11.8	11.2	11.8	30.0	32.3	34.2

$P_{CO_2}^* = 1.88 \cdot P_B$, which is derived from the typical molecular formula of microalgal biomass, CO_{0.48}H_{1.83}N_{0.11}P_{0.01} (Chisti 2007)

being in all cases, $P_{CO_2}^*$ slightly higher than P_{CO_2} . In this study microalgae specific CO₂ requirement has been also calculated (Table 4) ranging between 1.64 and 1.77 mg CO₂ per mg of suspended solids (Y_{CO_2}). Therefore the mentioned equation can be used as estimative method, without the need for a biomass elemental analysis and assuming a biomass carbon content of 50%, and hence average microalgae CO₂ requirements of 1.88 mg CO₂ per mg of microalgae generated. Since there is no influence of the N:P ratio on the carbon content in the microalgae biomass, P_{CO_2} capability is only related to the biomass productivity (P_b). Therefore, the maximum carbon dioxide biofixation rate is obtained at N:P between 13 and 22.

Lipid and Crude Protein Content

The lipid and crude protein content of *S. obliquus* under different N:P ratio are shown in Table 4.

The lipid content increases as the N:P decreases, reaching the maximum at R₁ (35% Lipids DW) and the minimum at R₃₅ (15% Lipids DW) (Table 4). This evolution is similar to that observed for the time needed to reach 90% of maximum biomass concentration (Table 2), so this trend can be related to the aging culture according to Hu *et al.* (2008) and Collins and Kalnins (1969). The age of the culture in this work is considered to be the difference between the hole time cultivation (264 h) and the time needed to reach the stationary phase (t_{90}) (Table 2). In Figure 3(a) it is represented the evolution of the lipids content as function of the culture aging. It can be appreciated that when the aging culture changes from 80 to 145 h, an increment of the lipid is observed, being constant for aging times above 145 h. As N:P ratio decreases not only the maximum biomass concentration is reached earlier (higher culture age at 264 h) but also the final N:P ratio, it is more extreme, for example in R₁ the N:P ratio at the end of the test was 0.3 ± 0.18 . Xin *et al.* 2010 tested *S. obliquus* in synthetic culturing media at 9 different N:P ratio, founding that at N:P ratio from 8.52 to 110 and at the same aging culture there were no great differences in the lipid content, ranging from 21 to 25%; and only at extreme value of N:P ratio an enhancement of the lipids biomass content was achieved, 31 and 55% lipid content at N:P of 4.26 and 221 respectively.

Crude proteins content is directly related to the nitrogen content in the biomass. Nitrogen content in biomass is presented in Table 4. Two groups can be easily distinguished.

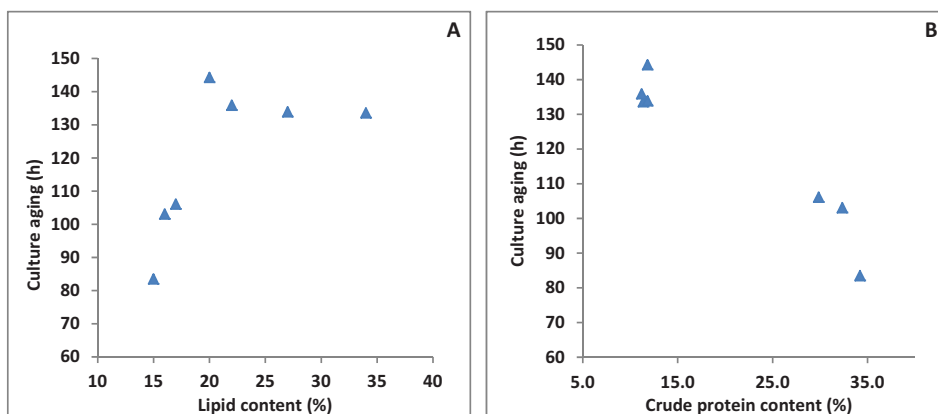


Figure 3 Evolution of lipid content (%) (3A) and crude protein content(%) (3B) as function of the aging culture (color figure available online).

Firstly, N:P from 1 to 9 with average nitrogen content of $1.88 \pm 0.23\%$. Secondly, those tests with N:P above 9 where the average nitrogen content was $5.03 \pm 0.20\%$. As for lipid content, the nitrogen content variation can be explained by the aging effect of the culture (Figure 3b). The shorter was the aging culture the greater the nitrogen content is (Figure 3b). And therefore the maximum crude protein content was reached in those tests with higher t_{90} (R_{35} , R_{22} and R_{13}) with an average of $32.13 \pm 2.18\%$ crude protein (Table 4) and the minimum in R_1 , R_3 , R_5 and R_9 with an average of $11.55 \pm 0.314\%$ crude protein

CONCLUSIONS

N:P variation of the wastewater showed no significant differences ($p < 0.05$) in terms of specific growth rate (μ_{\max}). The proposed batch productivity calculation method can reduce the dispersion of data related to the lag and stationary phase of the growth curves. The proper N:P ratio for achieving the optimum biomass productivity for *S. obliquus* ranged between 9 and 13. Related to the nutrient removal a great effect of the N:P ratio variations was observed, and for an efficient simultaneous nutrient removal, i.e. nitrogen and phosphorus, the proper N:P ratio ranged 9–13. In this interval, similar t_{10N} and t_{1P} were observed. Biomass lipids and crude protein content depends on the aging culture. As culture aging is higher lipid content increases and crude protein content decreases.

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APPENDIX

Equation 1:

$$\frac{dX}{dt} = \mu_{max} X \left(1 - \frac{X}{X_{max}} \right)$$

Equation 2:

$$X = \frac{X_0 X_{max} e^{\mu_{max} t}}{X_{max} - X_0 + X_0 e^{\mu_{max} t}}$$

Equation 3:

$$P_o = \frac{\Delta X}{\Delta t} = \frac{(X_{max} - X_0)}{(t_{\infty} - t_0)}$$

Equation 4:

$$t = \frac{1}{\mu} \text{Ln} \left(\frac{X \cdot (X_{max} - X_0)}{X_0 (X_{max} - X)} \right)$$

Equation 5:

$$t_{90} = \frac{1}{\mu} \text{Ln} \frac{9 \cdot (X_{max} - X_0)}{X_0}$$

Equation 6:

$$t_{10} = \frac{1}{\mu} \text{Ln} \left(\frac{1.1 \cdot (X_{max} - X_0)}{(X_{max} - 1.1 \cdot X_0)} \right)$$

Equation 7:

$$P_B = \frac{(X_{90} - X_{10})}{(t_{90} - t_{10})} = \frac{(0.9 \cdot X_{max} - 1.1 \cdot X_0)}{(t_{90} - t_{10})}$$

Equation 8:

$$P_B = \frac{\mu \cdot (0.90 \cdot X_{max} - 1.10 X_0)}{\text{Ln} \left(\frac{9 \cdot (X_{max} - 1.1 \cdot X_0)}{1.1 \cdot X_0} \right)}$$

Equation 9:

$$P_{\text{co}_2} = \%C \cdot P_B \left(\frac{\text{MW}_{\text{CO}_2}}{\text{MW}_C} \right)$$

Equation 10:

$$\text{C.I.} = \bar{X} \pm \left(\frac{\delta}{\sqrt{n}} \right)$$

Equation 11:

$$\frac{-dS}{dt} = K_2 \cdot S^2 + K_1 S + K_0$$